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Rubisco activity is associated with photosynthetic thermotolerance in a wild rice (*Oryza meridionalis*)

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Oryza meridionalis is a wild species of rice, endemic to tropical Australia. It shares a significant genome homology with the common domesticated rice *Oryza sativa*. Exploiting the fact that the two species are highly related but *O. meridionalis* has superior heat tolerance, experiments were undertaken to identify the impact of temperature on key events in photosynthesis. At an ambient CO_2 partial pressure of 38 Pa and irradiance of 1500 µmol quanta m^{-2} s^{-1}, the temperature optimum of photosynthesis was $33.7 \pm 0.8^{\circ}C$ for *O. meridionalis*, significantly higher than the $30.6 \pm 0.7^{\circ}C$ temperature optimum of *O. sativa*. To understand the basis for this difference, we measured gas exchange and rubisco activation state between 20 and 42°C and modeled the response to determine the rate-limiting steps of photosynthesis. The temperature response of light respiration (R_{light}) and the CO_2 compensation point in the absence of respiration (Γ^*) were determined and found to be similar for the two species. C3 photosynthesis modeling showed that despite the difference in susceptibility to high temperature, both species had a similar temperature-dependent limitation to photosynthesis. Both rice species were limited by ribulose-1,5-bisphosphate (RuBP) regeneration at temperatures of 25 and 30°C but became RuBP carboxylation limited at 35 and 40°C. The activation state of rubisco in *O. meridionalis* was more stable at higher temperatures, explaining its greater heat tolerance compared with *O. sativa*.

Introduction

At current atmospheric CO_2 concentrations, photosynthesis is limited by two prevailing factors, the fixation of CO_2 by the enzyme rubisco and the regeneration of the rubisco sugar-phosphate substrate, ribulose-1,5-bisphosphate (RuBP) through the Calvin Cycle (Farquhar et al. 1980, Long and Bernacchi 2003, Sharkey et al. 2007). RuBP carboxylation-limited photosynthesis (A_c) has been attributed to the catalytic turnover rate of rubisco, the affinity of the enzyme for CO_2 and the competitive effects of O_2 (Jordan and Ogren 1984, Brooks and Farquhar 1985, Spreitzer and Salvucci 2002, Salvucci and Crafts-Brandner 2004a). RuBP regeneration limitation (A_r) is associated with the membrane-bound reactions of electron transport activity, the production of ATP and NADPH and the various Calvin Cycle enzymes that regenerate RuBP (von Caemmerer 2000, Sharkey 2005, Yamori et al. 2011b). As both membrane stability (Armond et al. 1980, Gounaris et al. 1984, Havaux et al. 1996) and rubisco (Law and

Abbreviations – PPFD, photosynthetic photon flux density; RuBP, ribulose-1,5-bisphosphate.

Crafts-Brandner 1999, Crafts-Brandner and Salvucci 2000) are susceptible to changes in temperature, it is not surprising that photosynthesis is significantly constrained at high temperatures. One would expect strong evolutionary pressure for thermotolerance of photosynthesis in tropical plants and indeed, it is now becoming apparent that the temperature-dependent limitation in photosynthesis is species specific and highly dynamic (Hikosaka et al. 2006, Yamori et al. 2011a). In species such as spinach (*Spinacia oleracea*), wheat (*Triticum aestivum*) and black spruce (*Diceamariana*), rubisco is thought to limit photosynthesis at temperatures above the photosynthetic optimum (Sage et al. 2008, Yamori et al. 2010b). In other species including tobacco (*Nicotiana tabacum*), sweet potato (*Ipomoea batatas*) and rice (*Oryza sativa*), RuBP regeneration is thought to be the limitation at and above the temperature optimum (Cen and Sage 2005, Makino and Sage 2007, Yamori et al. 2010b). Not only does the limiting factor change over a given temperature range within species but habitat and acclimation to growth temperatures prior to experimentation can also affect the type of limitation (Hikosaka et al. 1999, Onoda et al. 2005, Yamori et al. 2006). Considering the increase in global surface temperatures and likelihood of more extreme weather events, including heat waves, understanding what drives the limitation in photosynthetic capacity is fundamental to identifying how plants will adapt to changes in climate.

One way of gaining a better understanding of the temperature-dependent limitations of photosynthesis is by comparing similar species from contrasting climatic regimes. With this in mind, we compared the responses to temperature of two *Oryza* species, *O. sativa* L. and *Oryza meridionalis* Ng. There are more than 20 species in the genus *Oryza* (Ge et al. 1999) and nine distinct genomes; *O. meridionalis* shares the same genome with the two cultivated species, *O. sativa* from Asia and *O. glaberrima* from Africa (Nishikawa et al. 2005, Duan et al. 2007, Sweeney and McCouch 2007). *O. meridionalis* has a distribution throughout northern Australia and West Papua (Ng et al. 1981, Lu and Silitonga 1999, Henry et al. 2010), in a subtropical, monsoonal environment. It has previously been established that growth of *O. meridionalis* has superior tolerance to brief periods of severe heat up to 45°C relative to *O. sativa* ssp. *japonica* (Scafaro et al. 2010). In the same study it was shown that many of the proteins that change in abundance after heat exposure are related to photosynthesis. In particular, rubisco activase and cpn60, a chaperone known to interact with rubisco activase (Salvucci 2008), had highly regulated responses to heat stress in *O. meridionalis*. The difference in temperature susceptibility between these two highly related species provides an opportunity to evaluate photosynthesis limitations and determine any contrasting mechanisms in the response of photosynthesis to temperature.

Materials and methods

Plant material and growth

Seeds of *O. meridionalis* Ng. were collected from a wild accession located in the Cape York Peninsula of Australia (15°41′57″S: 145°02′48″E). *O. sativa* ssp. *japonica* cv. Amaroo seeds were obtained from the Yanco Agricultural Institute (NSW Department of Primary Industries, NSW, Australia). All plants were grown in a glasshouse at 28 ± 3°C in pots with an organic soil mix and slow release fertilizer applied following manufacturer's instructions (Osmocote, Scotts, Baulkham Hills, Australia). All plants were well hydrated with approximately one-quarter of the bottom of pots submerged in water. Treatments were commenced after 45 days in the mid-tillering phase of development. All measurements were taken from healthy, young fully expanded leaves.

Determination of leaf mass, chlorophyll, rubisco content and rubisco activation state

For leaf mass per area (LMA), nitrogen, rubisco and chlorophyll determinations, a measured area of leaf (between 2 and 4 cm^2), mid-lamina, was removed and immediately frozen in liquid nitrogen and stored at −80°C. Samples were taken from three plants from separate pots. LMA was calculated after drying the leaf material for 72 h at 70°C and weighing the dried material. Oven-dried samples were also analyzed for nitrogen content using a Leco CHN-900 gas analyzer (Leco, St Joseph, MI). Chlorophyll content and calculated chlorophyll *a* and chlorophyll *b* concentrations were determined using the method of Porra et al. (1989).

Total rubisco catalytic sites were quantified by stoichiometric binding of ^{14}C-carboxy-arabinitol-P_2 (CABP-binding technique) according to Ruuska et al. (1998). Total sites were quantified using a CO_2-free extraction medium containing 50 m*M* Bicine-NaOH (pH 8.0), 10 m*M* $MgCl_2$, 15 m*M* $NaHCO_3$, 5 m*M* DTT, 2 m*M* EDTA, 1.5% (w/v) polyvinylpolypyrrolidone and 1.5% (v/v) protease inhibitor cocktail (Sigma, St Louis, MO).

For determination of rubisco activation state, leaf discs (0.5 cm^2) were floated in 25 m*M* MES-NaOH (pH 5.5), contained within a water-jacketed beaker. The solution was flushed with a continuous gas stream (271 mol mol^{-1} CO_2 in air) and gently stirred. The solution temperature was maintained by circulating water through the beaker jacket and monitored using a thermocouple placed just below the solution surface. After

30 min of illumination with 1000 µmol quanta m^{-2} s^{-1} at the temperatures indicated in the text, the discs were immediately frozen in liquid nitrogen. Frozen leaf discs were extracted in 100 m*M* Tricine-NaOH (pH 8.0, CO_2 free), 5 m*M* DTT, 1 m*M* EDTA, 5% polyvinylpyrrolidone (PVP_{40}), 6% polyethylene glycol 3350 (PEG_{3350}), 1 m*M* phenylmethanesulphonylfluoride and 10 µ*M* leupeptin. Rubisco activities were determined by incorporation of $^{14}CO_2$ into acid-stable products at 30°C (Barta et al. 2011), immediately upon extraction (initial activity) and after incubation of the crude extract for 3 min with 10 m*M* $MgCl_2$ and 10 m*M* $NaHCO_3$ to allow carbamylation of all available catalytic sites (total activity). Duplicate assays were conducted on each sample. Four samples were measured at each temperature and the activation state was calculated as the initial activity divided by the total activity multiplied by 100.

Gas-exchange measurements

Net CO_2 assimilation rates (A_n) were measured using a Li-COR LI-6400 gas-exchange system (LI-6400, LI-COR, Lincoln, NE) similar to that of Yamori et al. (2010a). The CO_2 dependence of photosynthesis was determined by varying CO_2 partial pressure of the LI-6400 reference chamber between 5, 10, 15, 20, 38, 55, 80, 100, 125 and 150 ± 2 Pa. All measurements were taken with a photosynthetic photon flux density (PPFD) of 1500 µmol quanta m^{-2} s^{-1}. Ambient temperatures of leaves were varied by adjusting the gas-exchange chamber until leaf temperatures of 25, 30, 35 and 40°C were reached. Gas-exchange measurements were commenced after the desired leaf temperature had been maintained for at least 30 min. At the end of the 40°C treatment, the light was turned off to determine dark respiration rates (R_{dark}). The leaf vapor pressure deficit was kept below 3.0 kPa at all temperatures by adding water to the Carbabsorb in the CO_2 scrubbing chamber of the LI-6400. The experiment was replicated on four occasions with plants belonging to separate pots on each occasion. The temperature optimum of photosynthesis (T_{opt}) was obtained by fitting quadratic curves to the individual plant replicates of temperature-dependent photosynthesis and the temperature at which maximum photosynthesis was reached was recorded (Fig. S1).

Modeling of photosynthetic limitations

The partial pressure of CO_2 at the site of chloroplast (C_c) was calculated from the relationship:

$$C_c = C_i - \left(\frac{A_n}{g_m}\right) \quad (1)$$

where C_i (Pa) is the intercellular CO_2 partial pressure and g_m (µmol m^{-2} s^{-1} Pa^{-1}) is the mesophyll conductance. g_m was taken from values calculated by Scafaro et al. (2011) for rice over the observed temperature range. From the subsequently generated $A-C_c$ curves, the rate-limiting step of photosynthesis was analyzed using the C3 photosynthesis model (Farquhar et al. 1980, von Caemmerer and Farquhar 1981). RuBP carboxylation-limited photosynthesis (A_c) was determined from:

$$A_c = \frac{V_{cmax}(C_c - \Gamma^*)}{C_c + K_c(1 + O/K_o)} - R_{light}, \quad (2)$$

where V_{cmax} (µmol m^{-2} s^{-1}) is the maximum rate of RuBP carboxylation, K_c (Pa) and K_o (kPa) are the rubisco Michaelis constants for CO_2 and O_2, respectively, and O (21 kPa) is the O_2 concentration.

K_c and K_o were taken from tobacco measured over the same temperature range by Bernacchi et al. (2002). R_{light} is the light respiration and Γ^* is the CO_2 compensation point in the absence of respiration. Both were calculated from the Laisk method (Laisk 1977), where the intersection of three independent $A-C_c$ curves with differing light irradiances corresponded to C^* (x-axis) and R_{light} (y-axis). C^*, the CO_2 compensation point in the absence of respiration at an intercellular CO_2 concentration was converted to chloroplast CO_2 concentration (Γ^*) using the mesophyll conductance (g_m) of rice determined by Scafaro et al. (2011) and the formula of von Caemmerer et al. (1994):

$$\Gamma^* = C^* + \frac{R_{light}}{g_m} \quad (3)$$

The C_a range used to generate the curves was 12, 10, 7.5 and 5 Pa and the light irradiances were 100, 200 and 400 µmol quanta m^{-2} s^{-1} (Fig. S2). As K_c and K_o were taken from tobacco, we also modeled the data using tobacco R_{light} and Γ^* measured by Bernacchi et al. (2001, 2002) to determine if the parameter measurements we made affected the model outcome (Fig. S3).

RuBP regeneration-limited photosynthesis (A_r) was determined from:

$$A_r = \frac{J_g(C_c - \Gamma^*)}{4C_c + 8\Gamma^*} - R_{light} \quad (4)$$

where J_g (µmol m^{-2} s^{-1}) is the chloroplast electron transport rate determined by gas exchange. Fitting of the model was performed using the software program GraphPad Prism (GraphPad Software Inc., San Diego, CA) and V_{cmax} and J_g were estimated from C_c observations below 20 Pa and above 30 Pa, respectively. The C_c values, at which the limitation of photosynthesis transitions from A_c to A_r (C_{trans}), were calculated using the

equation of von Caemmerer and Farquhar (1981):

$$C_{trans} = \frac{K_c(1 + O/K_o)J_g/4V_{cmax} - 2\Gamma^*}{1 - J_g/4V_{cmax}} \quad (5)$$

Statistical analysis

Differences between the two species in leaf properties and the temperature optimum of photosynthesis were assessed using two-sample *t*-tests (Table 1). A one-way ANOVA was used to assess temperature-dependent differences in photosynthesis. All graphs, curves and statistics were created using GRAPHPAD PRISM 5.0d software (GraphPad Software Inc.). All values given are means ± SE of three to four plant replicates, from different pots.

Results

Leaf properties, photosynthesis and respiration

The leaves of *O. meridionalis* were characterized by significantly lower LMA as well as lower contents of nitrogen, rubisco and chlorophyll than *O. sativa* (Table 1). There was 22, 23 and 27% less nitrogen, rubisco and chlorophyll per unit leaf area, respectively, and these differences were manifested in a lower photosynthetic capacity on an area basis in *O. meridionalis* compared with *O. sativa* (see below). There was no difference in the ratio of rubisco and chlorophyll to nitrogen, or the ratio of rubisco to chlorophyll between the two species, indicating that nitrogen partitioning was similar between *O. sativa* and *O. meridionalis*. Likewise, the chlorophyll *a*/*b* ratio was not significantly different. Further evidence that the investment of nitrogen into the components of photosynthesis is similar between the species is the J_g to V_{cmax} ratio at 30°C, which showed no species difference, and in rice J_g/V_{cmax} corresponds to the cytochrome *f* to rubisco ratio (Yamori et al. 2011a).

Table 1. Leaf properties of *Oryza sativa* and *Oryza meridionalis*. Chl *a*/*b*, chlorophyll *a* to chlorophyll *b* ratio; Chl/N, chlorophyll content per nitrogen content; J_g/V_{cmax}, ratio of chloroplast electron transport rate to maximum rate of RuBP carboxylation measured at 30°C; Rub/Chl, rubisco content per chlorophyll content; Rub/N, rubisco content per nitrogen content; T_{opt}, temperature optimum of net CO_2 assimilation rate. Asterisks indicate significant differences between *O. sativa* and *O. meridionalis* at $*P < 0.05$, $**P < 0.01$ and $***P < 0.001$. Values are means ± SE, $n = 3–4$.

	O. sativa	*O. meridionalis*
LMA (g m^{-2})	27.3 ± 1.6	23.1 ± 1.7***
Nitrogen (mmol m^{-2})	114 ± 2.7	87.6 ± 4.9***
Rubisco (μmol m^{-2})	6.70 ± 0.34	5.13 ± 0.40*
Chlorophyll (mmol m^{-2})	0.49 ± 0.07	0.36 ± 0.05**
Rub/N (μmol mol^{-1})	59.1 ± 3.0	57.8 ± 4.4
Chl/N (mmol mol^{-1})	4.31 ± 0.61	4.01 ± 0.54
Rub/Chl (mmol mol^{-1})	13.7 ± 0.7	14.3 ± 1.1
Chl *a*/*b* (mol mol^{-1})	3.04 ± 0.09	3.15 ± 0.11
J_g/V_{cmax} at 30°C	0.96 ± 0.03	0.92 ± 0.02
T_{opt} (°C)	30.6 ± 0.7	33.7 ± 0.8*

The temperature response of net CO_2 assimilation (A_n) varied between domestic *O. sativa* and wild *O. meridionalis*. At a C_a of 38 Pa and a PPFD of 1500 quanta m^{-2} s^{-1}, the temperature optimum of A_n (T_{opt}) for *O. sativa* peaked at 30.6 ± 0.7°C, similar to previous reports (Makino and Sage 2007, Yamori et al. 2010b) and was significantly lower ($t = 2.913$, d.f. = 4, $P = 0.0436$) than the T_{opt} for *O. meridionalis* at 33.7 ± 0.8°C (Table 1). At temperatures below or equal to 30°C, *O. meridionalis* had lower photosynthetic rates on a leaf area basis than *O. sativa* (Fig. 1A), but at temperatures of 35°C and above there was no difference in photosynthetic rate between the species. The fact that A_n decreased with an increase in temperature from 30 to 35°C in *O. sativa* but not in *O. meridionalis* indicated that, unlike in *O. sativa*, the photosynthetic performance of *O. meridionalis* was not compromised by moderately high temperatures. Furthermore, the fall in A_n with temperature in *O. sativa* was significant ($P = 0.006$), unlike *O. meridionalis* where there was no significant effect of temperature on A_n ($P = 0.262$) across the temperature range. Because of the lower leaf nitrogen content of *O. meridionalis*, A_n per unit of nitrogen for *O. meridionalis* matched the values for *O. sativa* at low temperatures and was greater than rates in *O. sativa* at temperatures above 35°C (Fig. 1B), indicating greater photosynthetic nitrogen use efficiency at the higher temperatures by the wild relative.

The different temperature response of A_n in the two species could not be attributed to a difference in CO_2 concentration in the leaves or to water relations, as C_i and stomatal conductance (g_s) were similar in degree and response for the two species (Fig. 1C, D) and to previously reported values (Scafaro et al. 2011) over the same temperature range. Furthermore, the difference in A_n between the species was not because of respiration as R_{light} and its response to temperature was similar between the species, both on an area and on a nitrogen basis (Fig. 2A, B). R_{dark} measured at 40°C was also similar between the species and substantially higher than R_{light} (Fig. 2C). The light to dark respiration ratio at 40°C was 0.47 and 0.53 for *O. sativa* and *O. meridionalis*, respectively, showing that in rice R_{light} is about 50% of R_{dark}. A lower R_{light} than R_{dark} is consistent with the ratios of R_{light}/R_{dark} found in many other plant species (Brooks and Farquhar 1985, Peisker and Apel 2001, Pinelli and Loreto 2003, Tcherkez et al. 2005, Atkin et al. 2006).

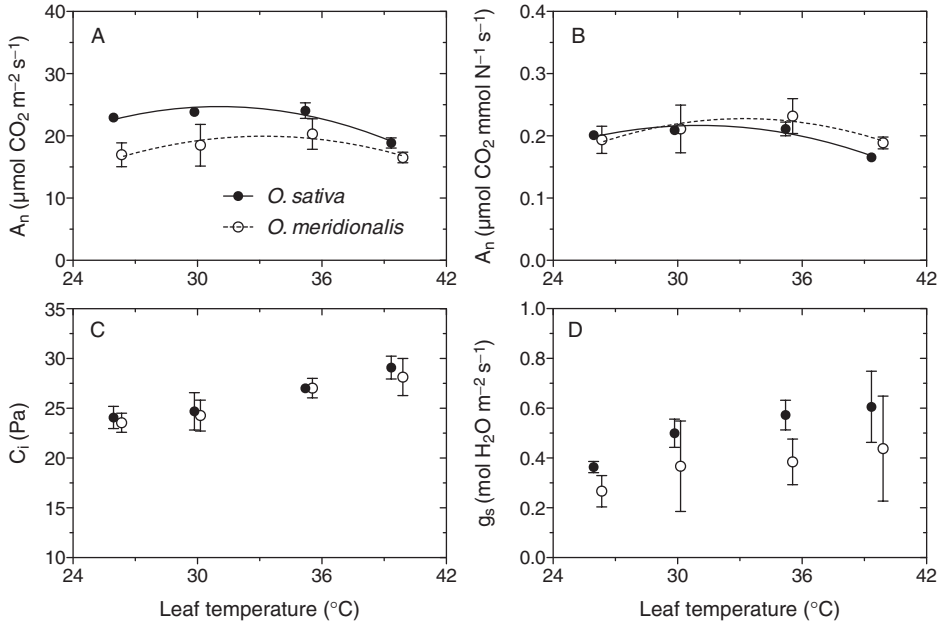

Fig. 1. Impact of leaf temperature on the net CO_2 assimilation rate (A_n), per area of leaf (A) and per unit of nitrogen (B), intercellular CO_2 partial pressure (C_i) (C) and stomatal conductance (g_s) (D) for *Oryza sativa* ssp. *japonica* (closed circles/solid line) and *Oryza meridionalis* (open circles/dashed line) at an ambient CO_2 concentration (C_a) of 38 Pa. All measurements were made at a PPFD of 1500 μmol quanta m^{-2} s^{-1}. Each point is the mean ± SE, of measurements made on fully expanded leaves from four plants belonging to separate pots. A_n values were fit with quadratic equations.

Modeled rate-limiting steps of photosynthesis

Measured Γ* and R_{light} showed that the temperature response of these variables are very similar between the two rice species (Fig. 3). Γ* and R_{light} values were also similar to measurements made in tobacco by Bernacchi et al. (2001, 2002), which is important considering we used the K_c and K_o of tobacco for modeling. The common temperature response of Γ* between tobacco and rice implies similar K_c and K_o (Brooks and Farquhar 1985) and the model parameters fit well with the observed A_n–C_c curves (Fig. 4). At a C_a of 38 Pa, photosynthesis was A_r limited at 25 and 30°C for both *O. sativa* and *O. meridionalis* (Fig. 4A–D). For temperatures of 35 and 40°C the limitation for both species changed to an A_c limitation (Fig. 4E–H). When the transition point of CO_2 partial pressure from A_r to A_c limitation (C_{trans}) is plotted against the recorded C_c at a C_a of 38 Pa, the similarity in photosynthetic limitations between the species is easily seen (Fig. 5). For our modeling, we used the K_c and K_o of tobacco with the Γ* and R_{light} measured in rice (Fig. 5 – method 1). However, when we used the Γ* and R_{light} measured in tobacco by Bernacchi et al. (2001, 2002) the two methods gave similar results with the same limitation predicted (Fig. 5 – method 2), thus affirming the reliability of the model.

Rubisco activity was measured at 271 μmol mol^{-1} CO_2 in air because of tank availability, however, rubisco activity is insensitive to CO_2 partial pressures ranging from 36 to 6 Pa at high light irradiance (Sage et al. 2002). By excising leaf disks the temperature of the leaf material was accurately controlled and similarity between heat inactivation of leaf disks and attached leaves has been reported (Crafts-Brandner and Law 2000). The rubisco activation state of both rice species showed different responses over the temperature range of 20–42°C (Fig. 6A). For the wild *O. meridionalis,* the activation state was not affected by temperature variations between 20 and 35°C and only decreased substantially at the highest measured temperature of 42°C. In contrast, the activation state of *O. sativa* was constant between 20 and 25°C, before decreasing progressively with increasing temperatures above 25°C. At the critical temperature of 35°C close to the T_{opt} of *O. meridionalis* but well above the T_{opt} of *O. sativa*, the rubisco activation state was 66 ± 4% for *O. meridionalis*, significantly higher ($P = 0.0386$) than the 54 ± 3% observed in *O. sativa*. As the total activity of rubisco differed between the two species, further analysis of the initial rubisco activity (Fig. 6B) showed *O. sativa* and *O. meridionalis* to be matched at temperatures reaching 30°C before *O. sativa* values fell below *O. meridionalis* for temperatures above 30°C.

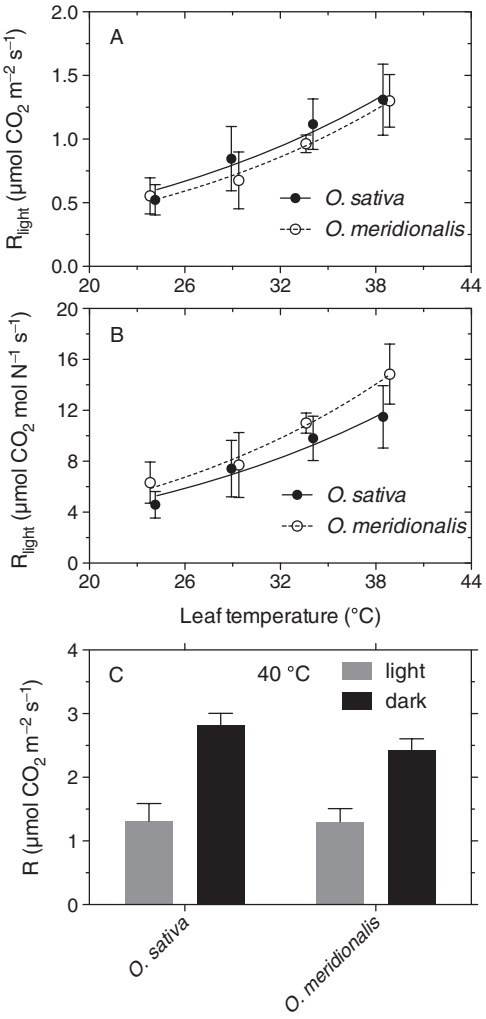

Fig. 2. The temperature response of R_{light} per area of leaf (A) and per unit of nitrogen (B) for *Oryza sativa* (closed circles/solid line) and *Oryza meridionalis* (open circles/dashed line) and R_{light} (gray) and R_{dark} (black) for *O. sativa* and *O. meridionalis* at 40°C (C). Each point is the mean ± SE, of measurements made on fully expanded leaves from four plants from separate pots. R_{light} values were fitted with an Arrhenius equation as per Bernacchi et al. (2001).

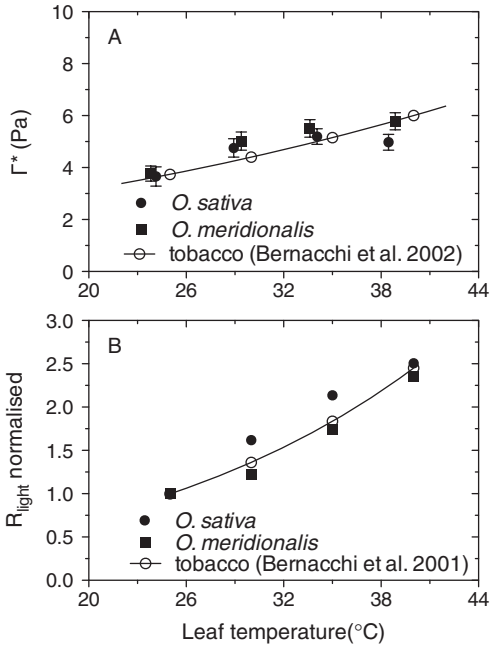

Fig. 3. The temperature response of the CO_2 compensation point in the absence of respiration (Γ*) (A) and R_{light} normalized to equal 1 at 25°C (B). Filled circles and squares represent values calculated by the Laisk method for *Oryza sativa* and *Oryza meridionalis*, respectively. Open circles/solid lines are the respective temperature functions derived from an Arrhenius equation (Bernacchi et al. 2001, 2002).

Discussion

The temperature response of A_n was different between *O. sativa* and *O. meridionalis,* with *O. meridionalis* having a greater tolerance to higher temperatures, particularly per unit of leaf nitrogen (Fig. 1). The heat-tolerant rice species *O. meridionalis* had a T_{opt} of 33.7°C, significantly higher than that of *O. sativa* and similar to the mean temperatures experienced in its natural habitat during the growing season. Data from the nearest weather station (15°50″S: 144°28″E), 60 km from the site of seed collection showed that the monthly mean daily maximum air temperatures for October, November and December were 33.7, 34.6 and 34.1°C, respectively (Australian Government Bureau of Meteorology; URL: http://www.bom.gov.au/climate/averages/tables/cw_028010.shtml). These temperatures, which exceed those typical of many rice-growing regions, may explain why *O. meridionalis* had a T_{opt} of 33.7°C and suboptimal photosynthetic capacity at lower temperatures. The photosynthetic temperature optima of many plants coincide with temperatures experienced in the corresponding natural habitats (Berry and Bjorkman 1980, Salvucci and Crafts-Brandner 2004b). The difference in the temperature response of photosynthesis between *O. sativa* and *O. meridionalis* was more pronounced than previously reported (Scafaro et al. 2011), however, the time exposed to each temperature was greater in this experiment and it is therefore likely that exposure time impacts upon A_n differentially between the rice species, with *O. meridionalis* having a greater ability to acclimate to high temperature.

The difference in the temperature response of A_n could not be attributed to differences in C_i, g_s or R_{light}, which were similar for both species (Figs 1C, D and 2). Furthermore, Scafaro et al. (2011) showed that *O. sativa* and *O. meridionalis* have a similar temperature response of g_m, which increases exponentially with temperature and results in a reduced chloroplast CO_2 drawdown at

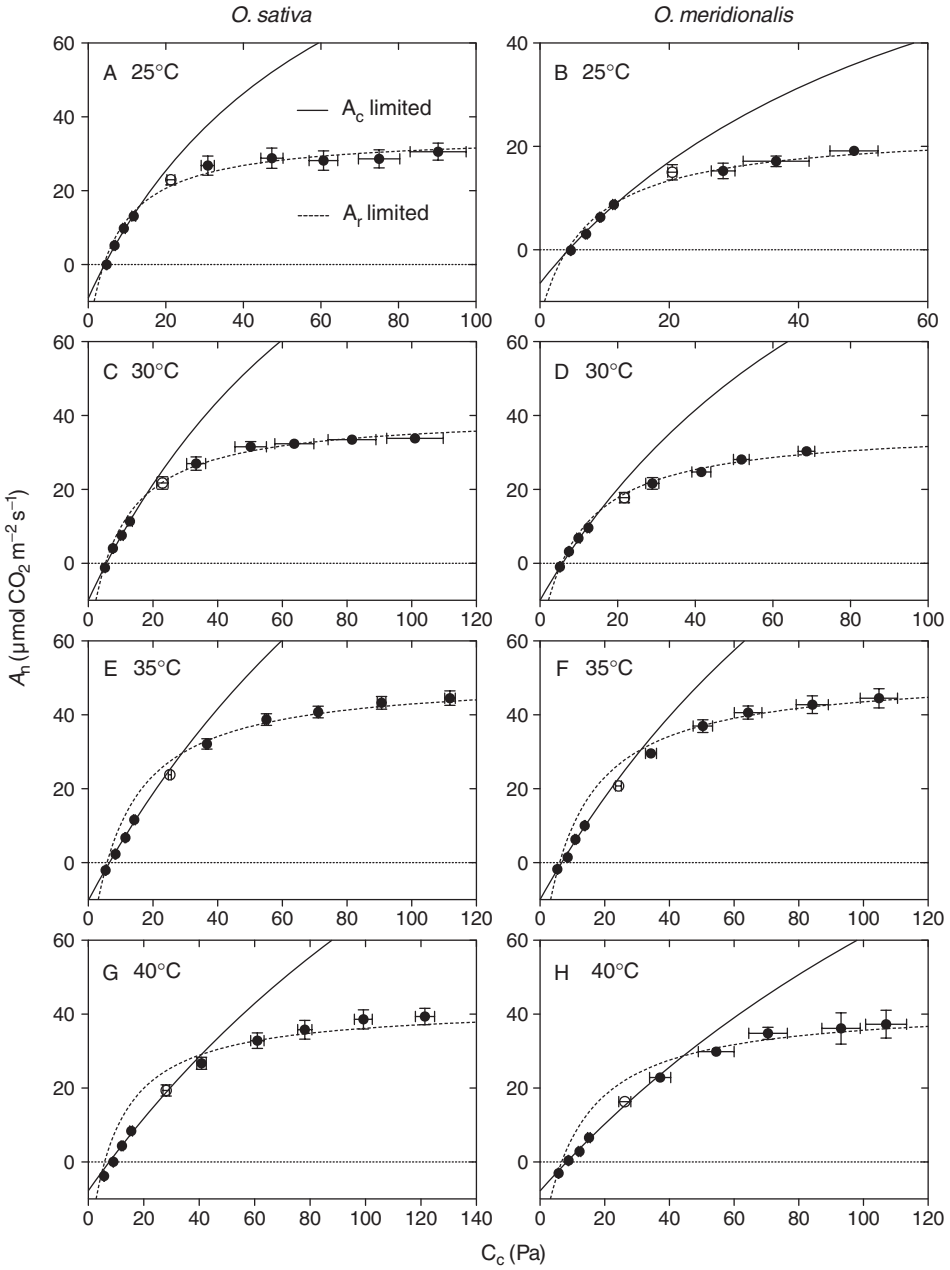

Fig. 4. Modeled photosynthesis limitations based on A–C_c curves measured at 25, 30, 35 and 40°C for *Oryza sativa* (A, C, E, G) and *Oryza meridionalis* (B, D, F, H). Solid lines indicate RuBP carboxylation-limited photosynthesis (A_c) and dashed lines indicate RuBP regeneration-limited photosynthesis (A_r), estimated from Eqns 2 and 4 in section Materials and methods. Filled circles correspond to the measured A–C_c rates over a range of C_a values and open circles represent measurements at a C_a of 38 Pa. Values are means ± SE of four pot replicates.

higher temperatures. Therefore, the different temperature response of A_n could not be attributed to g_m either, even though this has been cited previously as a limitation to photosynthesis in rice (Makino et al. 1994, Li et al. 2009). A similar R_{light} response between the two species is interesting as Atkin et al. (2006) found a close link between the temperature response of photosynthesis and respiration in the genus *Plantago*, with species from cold climates having a greater response to temperature for R_{light} than for photosynthesis, relative to closely related species from warmer climates. However, temperature acclimation of respiration is highly dependent on species (Atkin et al. 2005) and, unlike *Plantago*, it seems that in *O. sativa* and *O. meridionalis* A_n acclimates

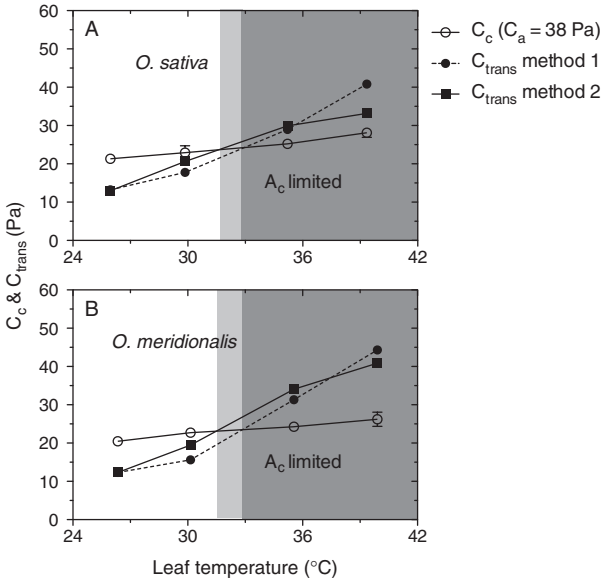

Fig. 5. Temperature limitations to photosynthesis at a C_a of 38 Pa, based on transition points from A_c to A_r (C_{trans}, calculated from Eqn 5 in section Materials and methods) for *Oryza sativa* (A) and *Oryza meridionalis* (B). Estimated C_c values (open circles/solid line) were plotted against C_{trans} values that were modeled using the Γ* and R_{light} parameters determined herein for rice (method 1, filled circles) and against C_{trans} values obtained from the model using Γ* and R_{light} parameters previously reported for tobacco by Bernacchi et al. (2001, 2002) (method 2, filled squares). Where C_{trans} values were greater than those of C_c, photosynthesis is limited by RuBP carboxylation (A_c). This limitation is depicted by a lighter shade where only one of the modeled C_{trans} curves is greater than C_c and by a darker shade when both models predict an A_c limitation.

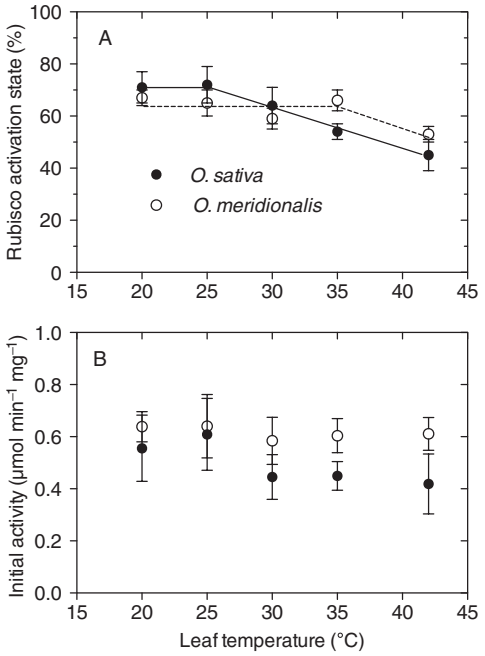

Fig. 6. The temperature response of rubisco activation state (A) and the initial activity of rubisco per unit of leaf protein (B) for *Oryza sativa* (closed circles/solid line) and *Oryza meridionalis* (open circles/dashed line). Lines were drawn to show the pattern of change in activation state with temperature. Values are means ± SE of four plants from separate pots.

independently of R_{light}, at least to short-term (minutes to hours) changes in temperature.

The temperature response of Γ* was the same for the two rice species and was similar to previous measurements in tobacco by Bernacchi et al. (2002) using an alternative method for determination of Γ* (Fig. 3). Considering that both Γ* and R_{light} are critical to the accuracy of the photosynthesis model (Pons et al. 2009) it is reassuring to find a similar temperature response of these variables between the different species, supporting the use of tobacco rubisco kinetics and providing confidence in the modeling. A similar temperature response of Γ* has been found in spinach (*S. oleracea*) using both values estimated by the Laisk method (Brooks and Farquhar 1985) and those based on the specificity factor of rubisco (Jordan and Ogren 1984, Yamori et al. 2006). Interestingly, Γ* did not increase from 35 to 40°C in either of the rice species and further measurements of Γ* at high temperatures are needed in other species, including spinach, where data are limited, to determine if this is an interspecific trait. Given the similarity in Γ* for rice, tobacco and spinach over the temperature range measured, it seems that the temperature response of Γ* is relatively well conserved between species. In vitro rubisco studies by Galmés et al. (2005) showed that the rubisco specificity factor (and therefore Γ*) and its temperature response varied among many non-cultivated species, particularly those from drier environments. However, when measured in vitro, differences in specificity factor because of drought were not found and a discrepancy between in vitro and in vivo measurements was noted (Galmés et al. 2006).

The rate-limiting step of photosynthesis was the same for both species (Fig. 4). Photosynthesis was A_r limited at 25 and 30°C, becoming A_c limited at 35 and 40°C. Other studies indicate an A_r limitation to photosynthesis in cultivated rice at similar temperatures (Makino and Sage 2007, Yamori et al. 2010b). It is thought that cold-sensitive species, including rice, are A_r limited over a wide range of temperatures, while cold-tolerant species seem to be A_c limited (Yamori et al. 2010b). In a similar study of Pima cotton (*Gossypium barbadense*) modeling indicated an A_r limitation over a similar temperature range, attributed to inhibition of electron transport capacity (Wise et al. 2004). One possible explanation for reduced rubisco activity with heat is downregulation

in response to thylakoid damage inhibiting electron transport (Sharkey 2005).

In C3 species including rice it is becoming apparent that plants with lower nitrogen and rubisco concentrations have an increase in the J_g/V_{cmax} ratio, which in turn leads to a shift from A_r to A_c limitation (Yamori et al. 2011a). In rice, Makino et al. (1994) found a sharper response of A_n to increased nitrogen content when plants were grown at higher temperatures. It is, therefore, likely that nitrogen availability and the subsequent rubisco content have a significant effect on the photosynthesis limitation of rice, a possible reason for the A_c limitation at high temperatures in this study, but not in others.

Although the temperature response of respiration, CO_2 conductance and water relations were not different between the rice species, a difference in the rubisco activation state at high temperatures was observed. Considering that temperature limitations to CO_2 fixation by rubisco are associated with a decrease in the activation state (Law and Crafts-Brandner 1999, Crafts-Brandner and Law 2000, Salvucci and Crafts-Brandner 2004a), the ability to maintain rubisco in an active state at high temperatures would support a higher level of photosynthesis in *O. meridionalis* under hot conditions. This ability to maintain rubisco in an active state would explain the higher T_{opt} of photosynthesis in *O. meridionalis*, because rubisco was the limiting factor of photosynthesis at high temperatures for both species and any difference in rubisco activation state would affect the photosynthetic rate. A small but significant fall in rubisco activity will reduce A_n marginally. However, even a small decrease in A_n can have a relatively large impact on growth, evident in growth rate comparisons between *O. sativa* and *O. meridionalis* at high temperatures (Scafaro et al. 2010). Interestingly, the amount of rubisco activase, the protein responsible for maintaining rubisco in an active form (Crafts-Brandner and Salvucci 2000, Salvucci and Crafts-Brandner 2004b), was found to be highly regulated in response to heat stress in *O. meridionalis* (Scafaro et al. 2010). The highly regulated response of rubisco activase to temperature in *O. meridionalis* is consistent with the involvement of this protein in maintaining rubisco in an active state at high temperatures.

Conclusions

The wild rice *O. meridionalis*, adapted to a warmer climate than *O. sativa*, had a higher temperature optimum of photosynthesis. The rate-limiting step of photosynthesis was the same for both species, changing from RuBP regeneration to RuBP carboxylation as temperature increased. When compared with *O. sativa*, the activation state of rubisco in *O. meridionalis* was more stable at high temperatures. Considering that photosynthesis is A_c limited at high temperatures, the ability to maintain rubisco in an active state at high temperature would improve photosynthetic capability, thus accounting for the higher T_{opt} of *O. meridionalis*.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Fig. S1. Determination of the optimum temperature of photosynthesis.

Fig. S2. Determination of Γ^* and R_{light} by the Laisk method.

Fig. S3. $A-C_c$ models using tobacco Γ^* and R_{light} parameters.

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